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Pex14p, a Peroxisomal Membrane Protein Binding Both Receptors of the Two PTS-Dependent Import Pathways

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Summary

Pex14p, an S. cerevisiae peroxin, is attached to the outer face of the peroxisomal membrane and is a component of the protein import machinery. Pex14p interacts with both the PTS1 and PTS2 receptors. It is the only known peroxisomal membrane protein that binds the PTS2 receptor and might thus mediate the membrane docking event of PTS2-dependent protein import. These results suggest that the two import pathways overlap and, furthermore, that Pex14p represents the point of convergence. Pex14p also interacts with two other membrane-bound peroxins including Pex13p, another binding protein for the PTS1 receptor. The data presented here are consistent with the idea of a common translocation machinery for both PTS-dependent protein import pathways in the peroxisomal membrane.

Introduction

Protein sorting plays a fundamental role in the biogenesis and maintainance of peroxisomes (Subramani, 1993). Proteins resident in the peroxisomal matrix and membrane are encoded by nuclear genes, synthesized on free ribosomes, and imported posttranslationally into the organelle. Genetic and biochemical evidence indicates that two pathways are involved in the sorting process of peroxisomal matrix proteins, corresponding to the existence of two distinct peroxisomal targeting signals, the C-terminal PTS1 and N-terminal PTS2 (Rachubinski and Subramani, 1995).

A second characteristic feature of the two import pathways for peroxisomal matrix proteins is that the recognition of the two targeting signals is performed by different PTS-specific receptors. Yeast and human cells selectively deficient in the PTS1 or PTS2 import pathways have been instrumental in the identification of the PTS receptors (Lazarow, 1993). The PTS1-specific receptor Pex5p (new nomenclature, see Distel et al., 1996) has been identified in four yeast species and in humans. In contrast, the PTS2-specific signal receptor Pex7p has so far only been found in Saccharomyces cerevisiae (Subramani, 1996).

The intracellular location of both targeting signal receptors is still a matter of debate. In a species- and experiment-dependent manner, these proteins have been predominantly or exclusively located in either the cytoplasm, the peroxisomal membrane, or even in the peroxisomal matrix (Rachubinski and Subramani, 1995).

This is reflected by three different models that are discussed for the functional roles of Pex5p and Pex7p. In the first model, the two PTS receptors bind their newly formed cargo proteins in the cytosol (or at the cytosolic face of the peroxisomal membrane) and direct them to the membrane-bound peroxisomal translocation machinery. After release of the cargo proteins, the receptors are free to cycle back to the cytosol (shuttle mechanism) (Marzioch et al., 1994; Wiemer et al., 1995; Elgersma et al., 1996; Erdmann and Blobel, 1996; Gould et al., 1996). In the second model, the cargo-loaded receptors cross the peroxisomal membrane together with their cargo proteins, these are unloaded in the matrix, and then the receptors shuttle back to the cytosol (extended shuttle mechanism) (Van der Klei et al., 1995; Dodt and Gould, 1996; Rehling et al., 1996). In the third model, the receptors are exclusively located inside the peroxisomes and promote import by pulling the proteins to be imported into the organelle (pulling mechanism) (Szilard et al., 1995; Zhang and Lazarow, 1996).

The two versions of a shuttle mechanism predict PTS receptor-specific docking sites at the peroxisomal membrane. In contrast, the pulling mechanism requires so far unknown PTS-specific receptors outside the peroxisome. The existence of PTS receptor-binding sites is supported by the very recent identification of a new peroxisomal integral membrane protein (Pex13p) that binds the PTS1 receptor with its cytosolic SH3 domain and, thus, could function as a peroxisomal docking site for PTS1-dependent protein import (Elgersma et al., 1996; Erdmann and Blobel, 1996; Gould et al., 1996). The cognate peroxisomal membrane protein that may serve as a receptor for the PTS2-dependent peroxisomal protein import has not yet been identified.

One of the key questions with respect to peroxisomal protein import to date is whether totally independent translocation machineries exist for PTS1- and PTS2-containing proteins or whether both pathways share a common translocation apparatus (Rachubinski and Subramani, 1995). The conjecture of a shared translocation machinery is supported by the many mutants that appear to be defective in the import of all peroxisomal matrix proteins (Kunau et al., 1993). Biochemical evidence for an overlapping of the pathways has not yet been provided.

In this paper, we report on Pex14p, the first membrane-bound peroxisomal protein that binds to the PTS2 receptor Pex7p. Thus, Pex14p may represent the functional docking site for PTS2-dependent protein import.

§ These authors contributed equally to this work.

A

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ScPex14p    MSDVVSKDRKA.LFDSAVSFLKDESIKDAPLLKKIEFLKSKGLTEKEIEI  49
            ||: .....:| |..|||.|| |:|| |.|| ||:|||.|||||:.|||
HpPex14p    MSQQPATTSRAELVSSAVEFLLDQSIADSPLAKKVEFLESKGLTQQEIEE  50

ScPex14p    AMKEPKKDGIVGDEVSKKIGSTENRASQDMYLYEAMPPTLPHRDWKDYFV  99
            |:..::.:.: :.. ..:... ...:    | : :|.||.||||||:
HpPex14p    ALQKARTGTVQASPSQQSVVPPRPPVPD....YYPSAPPLPERDWKDYFI  96

ScPex14p    MATATAGLLYGAYEVTRRYVIPNILPEAKSKLEGDKKEIDDQFSKIDTVL 149
            |||||||: ||.|:..:|||:|.|||..|..|||.|| .||.:| :::.:|
HpPex14p    MATATAGISYGVYQFVKRYVVPKILPPSKTQLEQDKAAIDHEFQRVESLL 146

ScPex14p    NAIEAEQAEFRKKESETLKELSDTIAELKQALVQTTRSREKIEDEFRIVK 199
            : :||:| || .|:... |.:.:|:.|:.: : .|. .. . |:.::.:|
HpPex14p    EKFEADQKEFYQKQEAKSKKIDETLQEVDEIINKTNEKNLNNEETLKYLK 196

ScPex14p    LEVVNMQNTIDK.FVSDNDGMQ.ELNNIQKEMESLKSLMNNRMESGNAQD 247
            ||: |:..|: | : |:...:. ||..::|:::.:|  :...  ...:|
HpPex14p    LEIENIKTTLLKTLDSQKATLNAELSAMEKQLQDIKFDIKTSGIAVAPQL 246

ScPex14p    NRLFSISPN.............GIPGIDTIPSASEILAKMGMQEESDKE 283
            .  | |..              .||....||| .::|.:     |.||:
HpPex14p    STPPSESTSRQSPAAEAKPKINLNIPPTTSIPSLRDVLSR.....EKDKD 291

ScPex14p    KENGSDANKDDNAVPAWKKAREQTIDSNASIPEWQKNTAANEIS....VP 329
             :.:| |. ::      :.|.|..::.  |||.|| .....: |    |:
HpPex14p    VNSDSIAQYEQ......RTANEKDVER..SIPAWQLSASNGGSSTTSGVA 333

ScPex14p    DWQNGQVEDSIP 341
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B

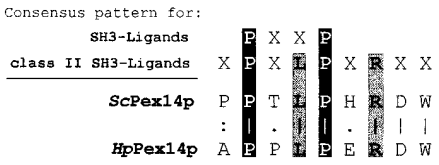


Figure 1. Analysis of ScPex14p Amino Acid Sequence

(A) Sequence comparison of Pex14p from H. polymorpha (HpPex14p) and S. cerevisiae (ScPex14p) using BESTFIT (EMBL, Heidelberg). Identities among amino acid residues are denoted by vertical bars. (B) Comparison of the consensus pattern for class II SH3 ligands in ScPex14p and HpPex14p. The minimal consensus for SH3 ligands is highlighted with black. Additional conserved amino acids in class II SH3 ligands are highlighted with gray.

Surprisingly, in addition to the interaction with the PTS2 recognition factor, Pex14p was found to interact with Pex5p, the PTS1 receptor. This observation suggests the overlapping of two import pathways, with Pex14p being the point of convergence. Moreover, Pex14p also interacts with two other membrane-bound peroxins including the SH3 domain protein Pex13p, the putative docking protein for PTS1-dependent protein import. We propose that these three peroxins are components of a common translocation machinery.

Results

Identification, Isolation, and Gene Deletion of *ScPEX14*

Current data libraries were screened with the deduced amino acid sequence of the Hansenula polymorpha *PEX14* gene (Komori et al., 1996). The analysis revealed an S. cerevisiae open reading frame (ORF) encoding a protein of 341 amino acids with a predicted molecular mass of 38.42 kDa. This ORF was termed *ScPEX14*. The deduced protein sequence of *ScPEX14* showed 35% sequence identity and 56% sequence similarity to the HpPex14p (Figure 1A) and was therefore considered a good candidate for the S. cerevisiae ortholog of *HpPEX14*. Protein database searches did not reveal significant sequence similarities between Pex14p and other proteins. Hydropathy analyses of the protein did not indicate the presence of membrane-spanning segments (data not shown). The proline-rich sequence motif in ScPex14p that is also found in HpPex14p (Figure 1B) corresponds well to the class II SH3 ligand consensus sequence (Mayer and Eck, 1995). Two different methods (Berger et al., 1995; Lupas, 1996) predict a coiled coil structure for residues 124–197 with a probability of 0.8–0.9 (data not shown). This could represent a structural element that allows oligomerization (Lupas, 1996).

Pex14p Is Constitutively Expressed

Two polypeptides with apparent molecular masses of 38 kDa and 30 kDa were detected in wild-type but not in Δ*pex14* yeast extracts with Pex14p-specific antiserum (Figure 2A). The 38 kDa protein corresponds well to the predicted molecular mass for Pex14p; the polypeptide of 30 kDa is most likely a Pex14p degradation product (Figure 2A, arrow).

In S. cerevisiae, growth on oleic acid results in a massive proliferation of peroxisomes accompanied by the induction of peroxisomal β-oxidation enzymes (Veenhuis et al., 1987). A time course of oleic acid induction of Pex14p and the β-oxidation enzyme thiolase (Fox3p) is shown in Figure 2B. In contrast to thiolase, Pex14p is constitutively expressed through the entire induction period.

Pex14p Is a Peripheral Peroxisomal Membrane Protein Tightly Bound to the Cytoplasmic Face of Peroxisomes

Double immunofluorescence microscopy localization of Pex14p and HA-tagged Pex11p (Erdmann and Blobel, 1995; Marshall et al., 1995) in wild-type cells showed colocalization of both proteins, consistent with a peroxisomal localization of Pex14p (Figure 2C). These immunofluorescence microscopy data were corroborated by results of subcellular fractionation studies. In sucrose density gradients of homogenates from oleic acid–induced wild-type cells, the majority of Pex14p comigrated with the peroxisomal marker enzymes catalase and thiolase at a density of 1.21 g/ml, which is characteristic of peroxisomes (Figure 2D). Moreover, analysis of Pex14p localization in oleic acid–induced wild-type cells by immunoelectron microscopy showed predominantly gold decoration of the peroxisomal periphery, suggesting that Pex14p is a peroxisomal membrane–associated protein (Figure 3A).

An organellar fraction isolated from spheroplasts of S. cerevisiae wild-type cells was subjected to extraction by low salt, high salt, and carbonate at pH 11 according to Erdmann and Blobel (1996). Pex14p was resistant to both low salt and high salt extraction but could be completely released from the membranes by treatment with carbonate at pH 11 (Figure 3B). These extraction properties distinguished Pex14p from three other peroxisomal proteins. Pex3p (formerly Pas3p, Höhfeld et al., 1991) was resistant to either means (Figure 3B), consistent with its being an integral membrane protein. The weakly membrane-associated peroxisomal protein

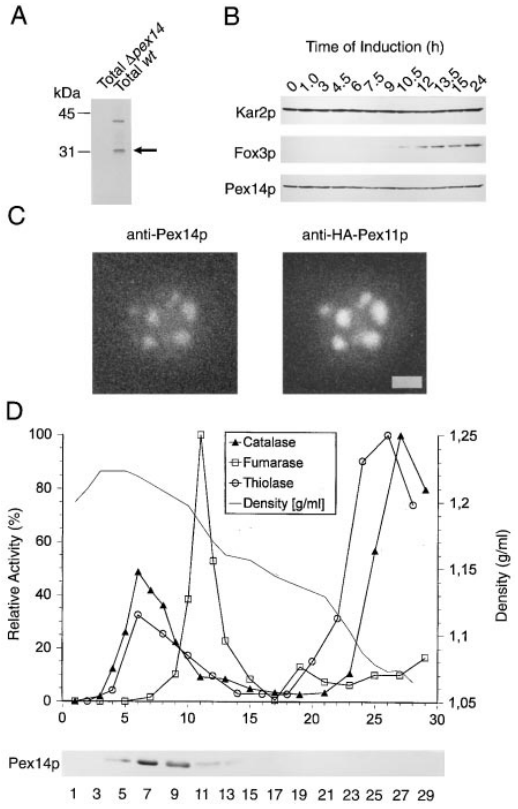


Figure 2. Immunological Detection, Oleic Acid Induction, and Subcellular Localization of Pex14p

(A) Immunological detection of Pex14p. Equal amounts of oleic acid–induced Δ*pex14* (total Δ*pex14*) and wild-type (total *wt*) homogenates (50 μg of protein) were subjected to Western blot analysis with rabbit antiserum against Pex14p. Molecular weight standards are indicated on the left. A degradation product of Pex14p is marked by an arrow.

(B) Time course of Pex14p induction during growth on oleic acid. Wild-type cells were precultured in 0.3 % SD and subsequently shifted to oleic acid-containing medium. At the indicated time points, whole cell extracts were prepared for immunological detection of Fox3p, Kar2p, and Pex14p. The amounts loaded per lane correspond to 0.3 mg of cells.

(C) Double immunofluorescence microscopy localization of thiolase and Pex14p in S. cerevisae wild-type cells. Wild-type cells expressing HA-tagged Pex11p were grown for 12 hr in YNO medium and processed for indirect double immunofluorescence microscopy using monoclonal antibodies against the HA epitope and rabbit antibodies against peroxisomal thiolase. One representative cell is shown in each photo. The bar indicates 0.5 μm.

(D) Immunological detection of Pex14p in fractions obtained by isopycnic 20%–54% sucrose density gradient centrifugation of cell homogenates from 12 hr oleic acid-induced wild-type cells. Relative amounts of peroxisomal marker enzymes catalase and thiolase and mitochondrial fumarase were monitored by activity measurements. Peroxisomes peaked in fraction 6 at a density of 1.21 g/ml, and mitochondria peaked in fraction 11 at a density of 1.18 g/ml. Equal volumes of each fraction were immunologically analyzed for the presence of Pex14p.

Pcs60p (Blobel and Erdmann, 1996) was resistant to low salt but was efficiently extracted by both high salt and carbonate treatment. Peroxisomal thiolase (Fox3p, Erdmann and Kunau, 1994), as expected for a protein localized in the peroxisomal matrix, was extracted by all

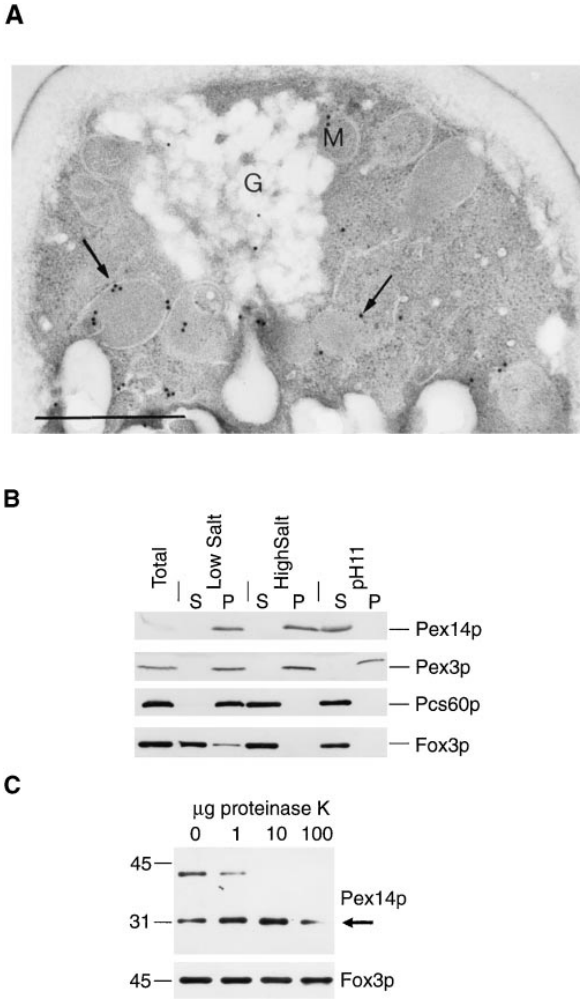


Figure 3. Subperoxisomal Localization of Pex14p

(A) Immunoelectron microscopy localization of Pex14p in whole cells. Immunogold labeling of the peroxisomal periphery is consistent with Pex14p being a peroxisomal membrane protein (arrows). Thin sections of lovicryl-embedded wild-type cells grown on oleic acid medium for 12 hr were processed for immunocytochemical analysis with polyclonal antiserum against Pex14p. (M), mitochondrion; (G), glycogen. Bars indicate 0.5 μm.

(B) Successive extraction of peroxisomes. 25,000 × g organelle pellets were prepared from oleic acid-induced wild-type cells and extracted by low salt, high salt, and carbonate treatment at pH 11.0. Extracted proteins were separated from the membranes by centrifugation. Equal proportions of pellet (P) and supernatant (S) fractions were analyzed by SDS–PAGE and Western blot analysis with specific antibodies against Pex14p, Pex3p, Pcs60p, and Fox3p. As Pex14p is extracted at pH 11.0 but not by high salt treatment, it behaves like a tightly associated peripheral membrane protein.

(C) Protease protection analysis of purified peroxisomes. A cell homogenate of wild-type cells was separated by sucrose density gradient centrifugation, and peroxisomal peak fractions were pooled. Equal amounts of the pooled peroxisomal fractions were incubated in the presence or absence of proteinase K for 10 min on ice. Samples were analysed by SDS–PAGE and Western blot analysis using antibodies against Pex14p and thiolase (Fox3p). The protease sensitivity of Pex14p suggests that the protein resides on the cytosolic face of the peroxisomal membrane. The observed Pex14p stable degradation product of 30 kDa is indicated by an arrow.

means. Thus, in resisting both low and high salt extraction but succumbing to carbonate treatment, Pex14p fulfills the requirements for a tightly bound peripheral membrane protein.

To determine the topology of Pex14p, isolated peroxisomes were incubated with proteinase K. Figure 3C shows that the 38 kDa protein band of Pex14p was extremely sensitive to the protease but remained at least partly stable when no protease was added. In contrast, the 30 kDa degradation product of Pex14p was not further degraded even in the presence of added protease, indicating that this product is either resistant to proteolysis per se or is protected by other proteins (Figure 3C). Since Pex14p does not span the peroxisomal membrane (Figure 3B), a protection of the 30 kDa peptide by compartmentation can be excluded. Thiolase remained resistant to protease digestion (Figure 3C) and was only subject to proteolytic degradation when detergent was added (data not shown). Taken together, the results of these experiments indicate that Pex14p is exposed to the cytoplasm. This conclusion is consistent with a number of other observations. In fractionation experiments, Pex14p remained highly sensitive to endogenous proteases, even in the presence of protease inhibitors, as indicated by its consistently observed 30 kDa degradation product (Figure 2A). Luminal peroxisomal proteins remained stable under these conditions. Moreover, Pex14p interacts with the SH3 domain of the integral membrane protein Pex13p (see below). As this part of the protein was reported to face the cytoplasm (Elgersma et al., 1996; Erdmann and Blobel, 1996; Gould et al., 1996), the in situ location of this interaction must be the cytoplasmic face of the peroxisome.

Δ*pex14* Cells Are Deficient in Peroxisomal Matrix Protein Import

A gene deletion of *PEX14* was constructed by replacing the genomic locus with the *LEU2* gene. Δ*pex14* cells grow well on YPD, SD, and ethanol but are unable to utilize oleic acid as single carbon source, a property characteristic of S. cerevisiae mutant strains affected in proteins essential for either peroxisome metabolism or biogenesis (oleic acid non-utilizing [onu]-phenotype; Erdmann et al., 1989). The ability to grow on oleic acid is restored by the introduction of genes expressing wild-type Pex14p (data not shown).

In contrast to wild-type cells, oleic acid-induced Δ*pex14* cells were characterized by the absence of morphologically detectable peroxisomes, thus indicating the requirement of Pex14p for proper peroxisome assembly.

Failure of Δ*pex14* cells to import matrix proteins of the PTS1 as well as the PTS2 variety was demonstrated by immunofluorescence microscopy, as shown in Figure 4A. Wild-type cells exhibited a peroxisome-characteristic punctate pattern when stained for the PTS1 protein Pcs60p (Blobel and Erdmann, 1996) or the PTS2 protein thiolase (Erdmann, 1994, Glover et al., 1994), shown in Figure 4A. In contrast, a diffuse staining pattern for both of these peroxisomal matrix proteins was observed in Δ*pex14* cells, indicating their mislocalization to the cytosol (Figure 4A). A punctate staining pattern for both proteins was restored in mutant cells expressing *PEX14*.

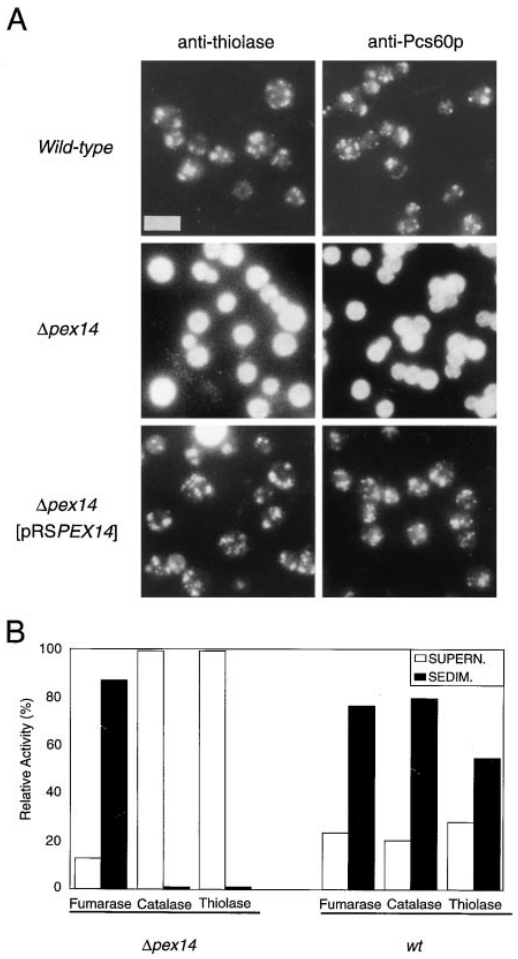


Figure 4. Δ*pex14* Cells Are Defective in Peroxisomal Matrix Protein Import

(A) Immunofluorescence microscopy localization of PTS2-containing thiolase and PTS1-containing Pcs60p in wild-type, Δ*pex14* mutant, and Δ*pex14* mutant cells expressing Pex14p from a single-copy plasmid. The bar indicates 5 μm.

(B) Subcellular distribution of peroxisomal and mitochondrial marker enzymes in oleic acid–induced Δ*pex14* and wild-type cells. After centrifugation of cell homogenates at 25,000 × g, the sediments and supernatants were assayed for peroxisomal catalase and thiolase as well as for mitochondrial fumarase activities.

To quantitate the import defect, the distribution of the peroxisomal matrix enzymes catalase and thiolase was determined by differential centrifugation of homogenates of both wild-type and Δ*pex14* cells (Figure 4B). The mitochondrial fumarase activity served as a control for the quantitation of organelle breakdown during homogenization. In wild-type cells, the majority of the peroxisomal and mitochondrial enzymes cosedimented with the organellar fraction. In Δ*pex14* cells, however, the peroxisomal matrix proteins were found exclusively in the soluble fraction. This observation is consistent with a general import defect for peroxisomal matrix proteins in Δ*pex14* cells.

Δ*pex14* Cells Retain Peroxisomal Membranes

If Pex14p is involved only in the topogenesis of peroxisomal matrix proteins, the localization of peroxisomal

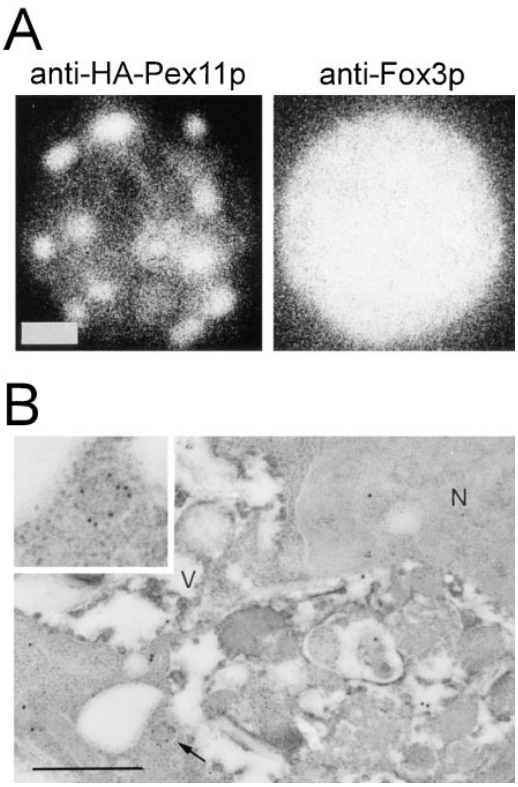


Figure 5. Δ*pex14* Cells Contain Peroxisomal Membrane Ghosts, Which Lack Peroxisomal Matrix Proteins

(A) Double immunofluorescence microscopy localization of thiolase and the peroxisomal membrane protein Pex11p in Δ*pex14* cells, expressing HA-tagged Pex11p. The bar indicates 0.5 μm.

(B) Double immunoelectron microscopy localization of Pex11p (10 nm gold) and thiolase (15 nm gold). (V), vacuole; (N), nucleus. The bar indicates 0.5 μm. The inset is a magnification of the vesicle pointed out by the arrow.

membrane proteins would be expected to remain unaffected in Δ*pex14* cells. In fact, double immunofluorescence microscopy localization of the peroxisomal membrane protein Pex11p (Erdmann and Blobel, 1995; Marshall et al., 1995) and the matrix protein thiolase in Δ*pex14* cells revealed a peroxisome-characteristic punctate pattern for Pex11p and a distribution of the thiolase label throughout the cytosol (Figure 5A). Similar cytoplasmic distribution was observed for the PTS1 protein Pcs60p (data not shown). Localization of Pex11p and the peroxisomal matrix enzyme thiolase by double immunoelectron microscopy (Figure 5B) confirmed the membrane localization of Pex11p (10 nm gold) and, thus, the existence of peroxisomal membrane ghosts in Δ*pex14* cells. Furthermore, the absence of the PTS2 protein thiolase (Figure 5B) within the lumen of the peroxisomal membrane ghosts of Pex14p-deficient cells confirms the import defect for this protein.

Pex14p Binds to Both PTS Receptors as well as to the Putative Docking Protein Pex13p

We used the two-hybrid system to detect in vivo protein–protein interactions between Pex14p and other peroxins reported to be directly involved in protein import. These included the PTS receptors Pex5p (Van der Leij et al., 1993) and Pex7p (Rehling et al., 1996; Zhang and Lazarow, 1996) and the putative docking protein for PTS1-dependent protein import, Pex13p (Elgersma et al., 1996, Erdmann and Blobel, 1996; Gould et al., 1996). Physical interactions of Pex14p with the peroxins was expected to result in β-galactosidase expression and His prototrophy of transformants. The results of these interaction studies are summarized in Figure 6A. Yeast cells coexpressing Pex14p and either Pex5p or Pex7p fused to the corresponding Gal4p domains were both His prototrophic (data not shown) and expressed β-galactosidase. These results demonstrate that Pex14p is capable of binding both PTS receptors in vivo. Moreover, coexpression of *PEX14–GAL4AD* and *PEX13–GAL4DB*, which encodes the cytosolic SH3 domain of Pex13p fused to the DNA-binding domain of Gal4p, resulted in both *lacZ* (Figure 6A) and *HIS3* (data not shown) transcription activation, indicating that Pex14p is also capable of binding the cytoplasmic domain of the peroxisomal integral membrane protein Pex13p. Furthermore, cells containing constructs with *PEX14* fused to both the Gal4p DNA-binding domain and the Gal4p activation domain also expressed both the *HIS3* and the *lacZ* genes, showing that Pex14p interacts with itself. The observed self-oligomerization of Pex14p might have its molecular basis in the suspected coiled coil element (see above). Pas9p (no Pex number has yet been given to Pas9p [Huhse, 1995]), another new peroxisomal peripheral membrane protein, was also shown to interact with Pex14p (Huhse and Kunau, submitted).

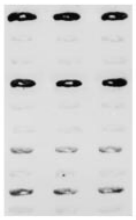
The controls included in Figure 6A show that coexpression of either of the fusion proteins, together with the respective Gal4p domains encoded by pPC86 and pPC97, did not support transcription activation of the reporter genes. Peroxins that did not interact with Pex14p in the two-hybrid system showed β-galactosidase activities in the range of the controls of Figure 6A and no staining in the filter assay (data not shown).

The strength of the interactions found with the two-hybrid system were quantitatively assessed, and the results did parallel the staining intensities obtained with the colony staining method. While Pex14p strongly interacts with the two PTS receptors, the binding to itself and especially to Pex13p was weak; however, it was still significantly above the activity level found in the controls (Figure 6A).

The Pex14p interaction with the two PTS receptors and Pas9p was independently confirmed by coimmunoprecipitation (Figure 6B). Three peroxins, Pex5p, Pas9p, and Pex14p, together with the PTS2 protein thiolase could be coimmunoprecipitated with mycPex7p from crude extracts of transformants expressing the fusion protein but not from control strains. In agreement with the observed weak interaction of Pex14p and Pex13p in the two-hybrid experiment (Figure 6A), the latter could not be detected in the immunoprecipitates. Attempts to identify PTS1 proteins were unsuccessful. Moreover, considering the relatively low amount of the PTS1 receptor found in the precipitates (Figure 6B) and the large number of PTS1 proteins known to exist, the amount of

A

Gal4-DB fused to	Gal4-AD fused to	β-galactosidase activity mU/mg protein
Pex7p	Pex14p	1867.3
Pex7p	-	10.7
-	Pex14p	9.2
Pex14p	Pex5p	453.1
-	Pex5p	8.6
Pex14p	-	9.4
Pex13p	Pex14p	19.8
Pex13p	-	6.7
Pex14p	Pex14p	64.4
-	-	7.3



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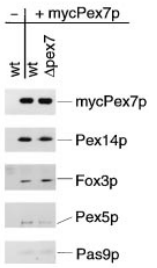

Figure 6. Pex14p Interacts with Other Peroxins

(A) Two-hybrid interactions of Pex14p. The amount of β-galactosidase activity in PCY2 double-transformands expressing the indicated combinations of Gal4p–peroxin fusion proteins is given in the table on the left. The β-galactosidase activity shown is the average of triplicate measurements for three independent transformants harboring each set of plasmids. The color intensity of these strains after the β-galactosidase filter assay is shown on the right.

(B) Coimmunoprecipitation of mycPex7p, Fox3p (thiolase), Pex5p, Pas9p, and Pex14p. Whole cell extracts of wild-type *(UTL-7A)* cells and of wild-type and Δ*pex7* cells expressing mycPex7p were immunoprecipitated with antibodies against the c-Myc epitope. Equal amounts of immunoprecipitates were separated on SDS–PAGE and subjected to immunoblot analyses using antibodies against Pex7p, Pex14p, Fox3p, Pex5p, and Pas9p.

each of these proteins might well be below the detection level.

Discussion

To date, three components of the import machinery for peroxisomal matrix proteins have been described. The two PTS receptors Pex5p and Pex7p serve as specific recognition factors for the peroxisomal targeting signals PTS1 (Van der Leij et al., 1993; Brocard et al., 1994) and PTS2 (Rehling et al., 1996; Zhang and Lazarow, 1996). The membrane-bound SH3 domain protein Pex13p is thought to function as a docking protein for PTS1-dependent protein import in S. cerevisiae (Elgersma et al., 1996; Erdmann and Blobel, 1996) and Pichia pastoris (Gould et al., 1996). Here, we describe Pex14p, a new membrane-bound component of the import machinery for peroxisomal matrix proteins, and demonstrate that it interacts with all three previously identified components. The data presented here suggest that Pex14p serves as a binding site at the peroxisomal membrane for both the PTS1- and PTS2-specific receptors and is thus a candidate for the predicted point of convergence for the two distinct protein import pathways. Moreover, Pex14p is proposed to be a key component of a common membrane-bound protein translocation machinery.

A Complex Interaction of Multiple Peroxins

We systematically applied the two-hybrid methodology in combination with coimmunoprecipitation to identify protein-protein interactions among peroxins of S. cerevisiae. The results, summarized in Figure 7A, indicate the existence of a complex network of peroxin interactions.

Remarkably, Pex14p was found to bind the PTS2-specific receptor Pex7p, suggesting that the discovery of this protein ends the long search for the peroxisomal membrane binding protein for PTS2-dependent matrix protein import. We previously proposed a shuttle mechanism for Pex7p function (Marzioch et al., 1994) that predicted the docking of a Pex7p-cargo protein complex at the peroxisomal membrane. Three lines of evidence suggest that Pex14p is a likely candidate for this peroxisomal docking protein. First, the results of the two-hybrid studies indicate that Pex14p interacts with Pex7p (Figure 6A). Second, detergent-solubilized Pex14p coimmunoprecipitated with both Pex7p and thiolase (Figure 6B). Third, the location of Pex14p at the outer face of the peroxisomal membrane is in accordance with the function of a binding protein (Figure 3).

These findings are difficult to reconcile with an exclusive intraperoxisomal location of the PTS2 receptor (Zhang and Lazarow 1996). Furthermore, Pex14p was found to interact not only with the PTS2 receptor Pex7p but also with the PTS1 receptor Pex5p (Figure 6) and with two additional peroxisomal membrane-bound peroxins, Pex13p (Figure 6) and Pas9p (Huhse and Kunau, submitted). Pex5p was recently shown to be predominantly located in the cytosol of S. cerevisiae (Elgersma et al., 1996), and the binding of Pex14p to Pex13p is mediated by the cytosolic SH3 domain of the latter. A proline-rich sequence that corresponds well to a typical class II SH3 domain binding motif is present within the

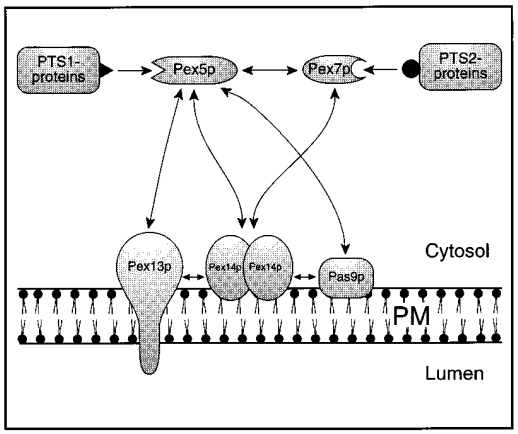

Figure 7. Pex14p Is the Point of Convergence of Both PTS-Dependent Protein Import Pathways

Pex5p has three binding partners, Pex13p, Pex14p, and Pas9p, at the outer face of the peroxisomal membrane. Of these proteins, only Pex14p interacts with the PTS2 receptor as well, suggesting that the overlapping of both PTS-dependent protein import pathways with Pex14p is the point of convergence. Pex14p also interacts with Pex13p, Pas9p, and itself. Taken together, these observations suggest that these interacting peroxins are components of a common translocation machinery for PTS1- and PTS2-dependent protein import into peroxisomes.

amino-terminal region of Pex14p (Figure 1B). Whether this motif is responsible for the observed Pex14p–Pex13p interaction remains to be tested. Quantitation of the Pex13-Pex14p two-hybrid interaction revealed a very low in vivo binding efficiency in comparison to the other Pex14p interactions. This could be due to either a "weak" or a transient in vivo interaction. In this context, it is interesting to note that Pex13p as well as Pex14p (Figure 6) are both able to bind the PTS1 receptor. Even more remarkable, the membrane-bound protein Pas9p, the third binding partner of Pex14p, is also able to interact with the PTS1 receptor (Huhse and Kunau, submitted). Whether all three of these membrane-localized peroxins, Pex13p, Pex14p, and Pas9p, compete for binding to the PTS1 receptor remains to be shown. An intriguing alternative suggests that the presence of multiple binding sites for the PTS1 receptor at the membrane may reflect the existence of a matrix protein import cascade in which a receptor-cargo protein complex moves from one component to the next (see below).

Pex14p, the Point of Convergence of PTS1- and PTS2-Dependent Protein Import?

After the discovery of the two distinct peroxisomal targeting signals for peroxisomal matrix protein import and the identification of the two corresponding PTS-specific receptors, recent research has focused on the question of whether the two newly discovered pathways are completely independent (Rachubinski and Subramani, 1995). The observation that most *pex* mutants of S. cerevisiae are characterized by defects in both PTS-dependent protein import pathways (Kunau et al., 1993) raised significant doubts and suggested that the two peroxisomal import pathways overlap. However, as long as the functions of the corresponding peroxins remain unknown, direct and indirect effects cannot be distinguished. Nevertheless, an early convergence of both pathways, for example, in a protein like Pex14p that binds both PTS receptors, could have been predicted by the genetic data. Of all *pex* mutants characterized in S. cerevisiae to date, only the two mutants defective in the PTS receptors are characterized by the PTS1- and PTS2-discriminating phenotype (Kunau et al., 1993), an observation which suggests that both pathways might converge right after the signal recognition step.

The existence of overlapping pathways was suggested by the identification of Pex13p, which is a component of the peroxisomal protein import machinery and was shown to be essential for both pathways (Elgersma et al., 1996; Erdmann and Blobel, 1996; Gould et al., 1996). However, Pex13p directly interacts only with the PTS1 receptor and not with the PTS2 receptor (Erdmann and Blobel, 1996), leaving open the question of how both pathways might converge. This riddle may now have been solved with the identification of Pex14p, a protein that interacts with both the PTS1 and the PTS2 receptors. Further support for the assumption that Pex14p is the point of convergence of both peroxisomal protein import pathways is provided by the fact that deficiency of Pex14p results in defects in both import pathways. Moreover, evidence that Pex14p might interact with Pex13p (Figure 6A) suggests an explanation as to why deficiency of this protein also affects both import pathways.

The discovery of Pex14p has far-reaching implications for our understanding of peroxisomal matrix protein import. Because of the limited data available, recent protein import models have left open the option that the two PTS receptors interact either with distinct PTS1- and PTS2-specific translocation complexes or with a common translocation complex (Rachubinski and Subramani, 1995). The existence of Pex14p as a membrane standing point of convergence for both pathways strongly favors the latter model for peroxisomal matrix protein import.

Distinct Pathways for Peroxisomal Matrix and Membrane Proteins

Recent evidence suggests the existence of independent pathways for the import of peroxisomal matrix proteins and the integration of peroxisomal membrane proteins (Erdmann and Blobel, 1996; Gould et al., 1996).

At the level of resolution provided by immunofluorescence and electron microscopy, matrix-deficient peroxisomal membrane "ghosts" were detected in Δ*pex14* cells. The ghosts were identified (Figure 5) by the presence of the peroxisomal membrane protein Pex11p (Erdmann and Blobel, 1995; Marshall et al., 1995). Obviously, the defect in matrix protein import caused by Pex14p deficiency had no effect on peroxisomal membrane formation. This result indicates that Pex14p is not required for targeting and insertion of peroxisomal membrane proteins. Thus, import of peroxisomal matrix proteins and integration of peroxisomal membrane proteins occur by different pathways. This finding seems to be unique to peroxisomes. Together with recent data indicating that the peroxisomal protein import machinery can accomodate folded proteins (McNew and Goodman, 1996), the existence of distinct import machineries for peroxisomal membrane and matrix proteins contributes to our current awareness of the complexity of peroxisomal protein import. It is becoming increasingly clear that these mechanisms are not just a plagiarization of what we know from other organelles but that new principles may apply.

Is There a Protein Translocation Complex at the Peroxisomal Membrane?

The multiple interactions of peroxins, although puzzling at first, may be a clue to the mechanism of peroxisomal protein import (Figure 7). Interaction of the PTS1 and PTS2 receptors suggests the possibility that they might form a heteromeric complex (Rehling et al., 1996) that is responsible for the cytoplasmic recognition and peroxisomal targeting of PTS-containing proteins. In S. cerevisiae, however, formation of this cytoplasmic receptor complex seems not to be a prerequisite for proper function, as absence of either receptor does not abolish performance of the other (Van der Leij et al., 1993; Marzioch et al., 1994; Zhang and Lazarow, 1995). Consequently, the observed in vivo interaction of the two PTS receptors in yeast might be optional rather than required. This seems to contrast with the situation in humans in which proper PTS2 receptor function has been

reported to depend on the presence of the PTS1 receptor (Dodt et al., 1995; Wiemer et al., 1995). The proposed cytosolic signal recognition in S. cerevisiae predicts the existence of receptor-specific docking sites at the peroxisomal membrane (Elgersma et al., 1996; Rehling et al., 1996). Published data for the PTS1 receptor (Elgersma et al., 1996; Erdmann and Blobel, 1996; Gould et al., 1996) and the data presented here for both receptors (Figure 6) indicate that these do indeed exist.

But why are there three potential binding sites for the PTS1 receptor on the three proteins Pex13p, Pex14p, and Pas9p, and do their interactions with each other reflect the existence of a heteromeric translocation complex at the peroxisomal membrane?

To answer the latter question, we will need to know whether the complex formed between these three membrane-bound peroxins is a dynamic structure based on transient interactions, or whether it is a stable entity. As a deficiency in any of the three components of the complex completely prevents import of matrix proteins into peroxisomes, they cannot represent redundant recognition systems at the outer face of peroxisomes but must each perform an essential role in the translocation process.

The existence of multiple binding sites for the PTS1 receptor on different components of the translocation machinery may reflect the existence of an import cascade. If PTS receptors do shuttle back and forth between the cytoplasm and the peroxisomal membrane or even the peroxisomal lumen (Rachubinski and Subramani, 1995, Dodt and Gould, 1996), property changes may be required. In this case, a successive delivery to different proteins might induce changes in the PTS receptors at the peroxisomal membrane. This might be necessary, for example, to allow the cargo protein to be released from the receptor for translocation into the peroxisome or to allow the receptors to shuttle back into the cytoplasm. In this context, it will be important to determine which of the three peroxins actually mediates the first step at the peroxisomal membrane (i.e., the docking event of the PTS1 receptor). Similar considerations can be made if the receptors have to reach the peroxisomal lumen for the translocation process. The latter is supported by the fact that the PTS2 receptor as well as the PTS1 receptor have been detected in the peroxisomal matrix (Subramani, 1996).

In conclusion, we have identified the peroxisomal Pex14p, a peroxin localized at the cytosolic face of the peroxisomal membrane that might function as the long-sought membrane receptor for PTS2-dependent protein import. Pex14p was shown to interact with the PTS1 and PTS2 receptors as well as with two other membrane-bound components of the peroxisomal import machinery for matrix proteins. The interaction with both PTS receptors suggests that this protein is the point of convergence of the PTS1- and PTS2-dependent pathways and thus provides strong evidence for the existence of a common translocation machinery for peroxisomal matrix proteins. The observed interactions between the three membrane-bound peroxins, Pex13p, Pex14p, and Pas9p, are consistent with the notion of a dynamic or stable protein translocation complex in the peroxisomal membrane.

Experimental Procedures

Strains, Growth Conditions, and General Methods

The yeast strains used in this study were S. cerevisiae wild-type *UTL-7A* (Marzioch et al., 1994), Δ*pex14* (*MATa, ura3-52, trp1/his3–11,15, ura2-52, pex14:: LEU2*), Δ*pex7* (Marzioch et al., 1994), Δ*pex14* [*PCS60*] and *UTL-7A* [*PCS60*] expressing Pcs60p (Blobel and Erdmann, 1996), Δ*pex14* [pRS*PEX14*] expressing Pex14p, Δ*pex14* [*PEX11*HA] and *UTL-7A* [*PEX11*HA] expressing HA-tagged Pex11p, and Δ*pex7* [myc*PEX7*] and *UTL7-A* [myc*PEX7*] expressing myc epitope–tagged Pex7p, *HF7c* (Clontech Laboratories, Inc.), and *PCY2* (Chevray and Nathans, 1992).

Yeast strains were grown at 30°C in YPD or in minimal medium (SD) as previously described (Erdmann et al., 1989; Erdmann et. al., 1991).

Whole yeast cell extracts were prepared by the method of Yaffe and Schatz (1984). Enzymatic modification of DNA, fragment purification, and bacterial transformation were performed essentially as described by Ausubel et al. (1989). Yeast transformations (except for introduction of the two-hybrid vectors into strain *HF7c*) were carried out according to Gietz and Sugino (1988). Total protein was measured by the BCA method (Pierce) using bovine serum albumin (BSA) as standard.

Cloning of the *PEX14* Gene and Oligonucleotides

The *PEX14* ORF was amplified from yeast genomic DNA by PCR using oligonucleotides KU107 and KU109. The amplification product was subcloned into EcoRV-digested pBluescript SK(+) (Startagene, La Jolla, CA) to create pSK*PEX14*. A 2237 bp DNA fragment containing the *PEX14* ORF as well as 601 bp of the 5′ and 628 bp of the 3′ noncoding region of *PEX14* was amplified using primers KU108 and KU110 and subcloned into pUC57 (MBI Fermentas, Lithuania), giving rise to pUC57*PEX14*. For complementation studies, the 2.2 kb fragment of pUC57*PEX14* was subcloned into the yeast *CEN* plasmid pRS316 (Sikorski and Hieter, 1989) to create pRS316*PEX14*. EcoRI and StuI sites in oligonucleotide KU107 were used for in-frame fusion to either glutathione S-transferase in plasmids pGEX-4T-1 or GAL4 domains of pPC86 and pPC97.

Oligonucleotides used in this study are as follows: KU107 (5′ GGA ATTCGAG GCCTTATGAG TGACGTGGTC AGT 3′), KU108 (5′ GGAT CCACTA GTAGGCCTCC CGCCATAATT GCA 3′), KU109 (5′ GGAAC TCCTT GCTATTGGTA CCCGGGATCC CG 3′), KU110 (5′ CGGGAT CCCG GGTACCAATA GCAAGGAGTT CC 3′) KU125 (5′ GGAATTC AGA TCTATGAGTG ACGTGGTCAG T 3′), p10-3 (5′ CAGGAATTCG GATCCCATAT GGACGTAGGA AGTTG C 3′), and p10-5 (5′ CAGCT CGAGA AACGAAAATT CTCCTTT 3′).

Construction of a *pex14* Null Allele

The SmaI and HindIII fragment of pJJ282 (Jones and Prakash, 1990) containing the *LEU2* gene was introduced into the *PEX14* ORF by using the internal HincII and HindIII sites, resulting in pPR23/2. This plasmid contained the *LEU2* gene flanked by bp 1–124 and 700–1026 of the *PEX14* coding sequence. The linearized plasmid was transformed into S. cerevisiae wild-type *UTL-7A*. Correct targeting of the *LEU2* gene was confirmed by PCR using primers KU107 and KU109.

Antibodies, Immunoblots, and Coimmunoprecipitation

The *PEX14* ORF was excised from pSK*PEX14* at the primer-derived EcoRI and vector-derived NotI sites and ligated into pGEX-4T-1 (Pharmacia, Uppsala, Sweden). The resultant plasmid pGEX4T1–*PEX14* was introduced into E. coli *TG1*. The transformants were induced with 0.1 mM IPTG, and the glutathione S-transferase–Pex14p fusion protein was purified according to the instructions of the manufacturer (Pharmacia, Uppsala, Sweden).

For generating antibodies against Pex5p, the *PEX5* ORF was amplified by PCR using plasmid pSK*PEX5*T (Erdmann and Blobel, 1996) and oligonucleotides p10-3 and p10-5. The PCR product was digested with EcoRI and NdeI and cloned into pET-21b (Novagen, USA), leading to plasmid pET-p10. The plasmid was transformed into E. coli BL21-DE3, resulting in IPTG-inducible expression of HIS6-tagged Pex5p. The tagged Pex5p was purified by affinity chromatography on a Ni-nitrilotriacetic acid resin (Quiagen, Hilden, Germany) according to the protocol of the manufacturer. Rabbit polyclonal antibodies to GST-Pex14p and HIS6-tagged Pex5p were

produced by Eurogentec (Seraing, Belgium) according to standard methods (Harlow and Lane, 1988).

Anti-thiolase (Fox3p), anti-Kar2p, anti-Pcs60p, anti-Pex3p, and anti-Pas9p antibodies have been described previously (Rose et al., 1989; Höhfeld et al., 1991; Erdmann and Kunau, 1994; Huhse, 1995; Blobel and Erdmann, 1996). Anti-rabbit or anti-mouse IgG-coupled HRP (Amersham Corp., Illinois) was used as the second antibody, and blots were developed using the ECL system (Amersham Corp., Illinois). Western blot analyses were performed according to standard protocols (Harlow and Lane, 1988).

Coimmunoprecipitation experiments were performed as described (Rehling et al., 1996), with the exception that the 35,000 × g sedimentation step was omitted.

Cell Fractionation

Spheroplasting of yeast cells, homogenization, and differential centrifugation at 25,000 × g of homogenates were performed as described previously (Erdmann et al., 1989).

For subfractionation by isopycnic sucrose density gradient centrifugation, cell lysates were loaded onto a linear 20%–53% sucrose density gradient. Centrifugation, fractionation of the gradient, and preparation of the samples for SDS-PAGE were carried out as described by Höhfeld et al. (1991).

Fractions were assayed for 3-oxoacyl-CoA thiolase (EC 2.3.1.16), catalase (EC 1.11.1.6), and fumarate hydratase (fumarase: EC 4.2.1.2) according to published procedures (Veenhuis et al., 1987).

The suborganellar localization of proteins was determined by extraction of 25,000 × g organellar pellets with low salt (10 mM Tris/HCl [pH 8.0], 1 mM PMSF), high salt (10 mM Tris/HCl [pH 8.0], 500 mM KCl, 1 mM PMSF), or pH 11 buffer (0.1 M Na_2CO_3, 1 mM PMSF) according to Erdmann and Blobel (1996).

Protease Protection Assay

Peroxisomal peak fractions from a sucrose density gradient were pooled and diluted five times in gradient buffer. Peroxisomes were sedimented at 25,000 × g for 30 min (Erdmann et al., 1989) and subsequently resuspended in homogenization buffer supplemented with 50 mM KCl but without protease inhibitors. Equal amounts of isolated peroxisomes were incubated with increasing amounts of proteinase K for 10 min on ice. Protease was inactivated immediately after the incubation with 4 mM PMSF, proteins were precipitated with TCA, and samples were processed for SDS-PAGE.

Electron Microscopy and Immunoelectron Microscopy

For electron microscopy, intact yeast cells were fixed with 1.5 % $KMnO_4$ and prepared as described by Erdmann et al. (1989). Immunogold labeling was performed as described previously by Douma et al. (1985).

Immunofluorescence Microscopy

Immunofluorescence microscopy was performed according to Erdmann (1994). Solutions (6 μg/ml) of CY3-conjugated donkey anti-mouse IgG and FITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) were used for detection.

Two-Hybrid Methodology

The 1.1 kb EcoRI/SacII fragment from pSK*PEX14*, containing the complete *PEX14* ORF, was subcloned in EcoRV/SacII-digested pPC86 (Chevray and Nathans, 1992). The resulting plasmid was designated pPC86-*PEX14*. For the construction of pPC97-*PEX14,* the *PEX14* ORF was excised from pPC86-*PEX14* with SalI/SacII and subcloned into pPC97 (SalI/SacII). Constructions of Gal4-DB-Pex7p, Gal4-AD-Pex5p, and Gal4-DB-Pex13p have been described previously (Erdmann and Blobel, 1996; Rehling et al., 1996).

Cotransformation of two-hybrid vectors into strain *HF7c* (Clontech Laboratories, Inc.) was performed according to the protocols of the manufacturer. Transformed yeast cells were plated on SD synthetic medium without tryptophane and leucine. β-galactosidase filter and liquid assay as well as the test for *HIS* prototrophy were performed according to Rehling et al. (1996).

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